Development of Anti-Bovine TNF-α mAb and ELISA for Quantitating TNF-α in Milk After Intramammary Injection of Endotoxin

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ABSTRACT

Murine mAb reactive with recombinant bovine tumor necrosis factor-α (r-boTNF-α) were produced. An ELISA using murine mAb and rabbit polyclonal antibodies, each reactive with r-boTNF-α to sandwich bovine TNF-α was developed.

Secretion of TNF-α in quarter milk increased 1 h after injection of 0.1 mg (four cows) or 0.5 mg (four cows) *Escherichia coli* lipopolysaccharide (LPS) into a mammary quarter, peaked 1 to 5 h later, and returned to control levels in 24 h. There were no differences in body temperature, SCC, TNF-α, and blood leukocyte responses between 0.1 and 0.5 mg of LPS. To determine effects of repeated injections of LPS into the same udder, a second injection of 0.1 mg of LPS into the same quarter (two cows) 24 h after the first injection produced a strongly attenuated TNF-α response. However, a normal TNF-α response was observed when LPS was injected into a contralateral quarter (two cows) 24 h after the first LPS injection. Leukocyte counts in blood decreased and body temperature increased substantially after each injection of LPS. Quarter milk SCC increased 200-fold 8 to 12 h after the LPS injections. It would appear that these changes were not regulated by TNF-α secretion because the changes were also similar after the second injection of LPS into the same mammary quarter.

(**Key words:** tumor necrosis factor-α, endotoxin, lipopolysaccharide, mastitis).

Abbreviation key: APR = acute-phase response, **IL-1** = interleukin-1, **LBP** = LPS binding protein, **LPS** = lipopolysaccharide, **r-boTNF-α** = recombinant bovine tumor necrosis factor-α, **TNF-α** = tumor necrosis factor-α.

INTRODUCTION

The acute phase of the inflammatory response refers to the wide-ranging physiological changes that are initiated immediately after an infection or physical trauma has occurred. The mammalian acute-phase response (**APR**) is characterized by fever, changes in vascular permeability, along with changes in the biosynthetic, metabolic, and catabolic profiles of many organs. The response is initiated and coordinated by a large number of diverse inflammatory mediators, which include cytokines, anaphylatoxins, and glucocorticoids (Steel and Whitehead, 1994). Regardless of the initiating event, the macrophage or monocyte is usually the cell that elicits the APR cascade (Baumann and Gauldie, 1994). Activated macrophages release a broad spectrum of mediators, of which cytokines of the interleukin-1 (**IL-1**) and tumor necrosis factor (**TNF**) families appear to be uniquely important in initiating the next series of reactions. These early, or "alarm," cytokines have pleiotropic activity and act both locally and distally. At the reactive site, IL-1 and TNF act on stromal cells including fibroblasts and endothelial cells, to cause the release of a secondary wave of cytokines. This secondary wave augments the homeostatic signal and initiates the cellular and cytokine cascades that are involved in the complex process of the APR. As a result, molecules that emerge from the local tissue site are highly chemotactic for neutrophils and monocytes. After migration, leukocytes begin to synthesize and release their own particular set of cytokines within the target tissue (Cassatella, 1995). The endothelium plays a critical role in communicating between the site of tissue inflammation and circulating leukocytes. Once again, cytokines such as IL-1 and TNF exert profound effects on progressing APR. Endothelial cells are induced to undergo major changes in surface expression of important adhesion and integrin molecules, which interact specifically with neutrophils and other circulating leukocytes to slow their rate of flow, initiate transendothelial passage, and allow subsequent migration into the tissue (Kishimoto

and Anderson, 1992). Thus, the APR follows a sequence of events in which macrophages and platelets are activated, alarm cytokines are released, adjacent stroma is recruited to secrete further chemotactic peptides, and leukocytes accumulate in the affected tissue.

Two systemic physiological responses in particular are regarded as being associated with acute inflammation. The first involves the generation of the febrile response. Three cytokines that are released from the site of tissue injury, IL-1, TNF, and IL-6, are considered to regulate the febrile response (Dinarello et al., 1991). The second involves alterations in metabolism and gene regulation in the liver. The liver response is characterized by the increased concentrations of the acute plasma proteins (Steel and Whitehead, 1994).

Lipopolysaccharide (**LPS** or endotoxin), a complex glycolipid from the outermost membrane of Gram-negative bacteria is a potent trigger of APR. The clinical syndrome of Gram-negative bacterial septicemia appears to result primarily from excessive stimulation of host immune system by LPS (Wright et al, 1990). Lipopolysaccharide induction of cytokine release, particularly TNF and IL-1, is probably the central event (Kishimoto and Anderson, 1992; Lynn and Golenbock, 1992). Lipopolysaccharide stimulates the cells by ligating specific membrane receptors (Wright, 1991), of which particularly important in cytokine release appears to be CD14, because anti-CD14 monoclonal antibodies block TNF release from leukocytes (Wright et al., 1990; Dentener et al., 1993; Haziot et al., 1993). CD14 is known to be present on human monocytes and macrophages and to a lesser degree, on neutrophils (Jayaram and Hogg, 1989; Landmann et al., 1991).

We have recently shown (Paape et al., 1996) that, as in human blood, the majority of monocytes in bovine blood are CD14 positive, while only very few neutrophils bear this receptor on their membrane. On the contrary, the majority of bovine mammary neutrophils, but the minority of bovine mammary mononuclear leukocytes are CD14 positive in milk from normal mammary glands. However, after an intramammary injection of LPS, there is a rapid migration of a very large number of neutrophils from the circulation to the mammary gland and the percentage of mammary neutrophils expressing CD14 dramatically decreases. Why the vast majority of migrated neutrophils remain incompetent to express this receptor is presently unknown. We have, however, shown that a large intracellular pool of CD14 exists in bovine blood neutrophils that is capable of becoming translocated to the cell surface over time during incubation in skimmed milk in vitro. In the present work we wanted to extend these observations by measuring the levels of TNF in blood and milk and observing the migration of neutrophils and the systemic

fever reaction after repeated intramammary injections of LPS.

MATERIALS AND METHODS

Preparation of mAb to Bovine TNF-α

Four female BALB/cByJ mice (Jackson Laboratories, Bar Harbor, ME) were each injected intraperitoneally with 0.1 mg of recombinant bovine TNF-α (**r-boTNF-α**, generously provided by Serge Martinod, CIBA-GEIGY, Basel, Switzerland) in 0.5 ml of 0.01 M phosphate buffer 0.85% saline, pH 7.4 (PBS). Three injections of r-boTNF-α were made per mouse; the first two injections were 4 wk apart, and the third was 6 wk after the second. Blood samples were collected after the second and third injections, and the sera were tested for antibodies to r-boTNF-α using an ELISA. Three days after the last injection, one mouse was killed and the spleen removed. The splenic cells were fused to Sp2/0-Ag cells (ATTC CRL 1581) by a modification of the Kohler and Milstein (1975) method as described by Van Deusen and Whetstone (Van Deusen and Whetstone, 1994). Fifteen 96-well plates were seeded with 5×10^5 cells/ml, and ten 96-well plates were seeded with 2.5×10^5 cells/ml. The primary and cloned cell lines were screened using an ELISA. Of the 1050 primary hybrids, 38 with the highest production of antibody (absorbance > 1.0 in ELISA) were kept. Of those 38 cell lines, 11 stopped secreting the antibody to r-boTNF-α, and nine died. Two primary cell lines (2C4 and 22D3) were cloned by limiting dilution; final concentration was three cells/ml, without feeder cells. All hybridoma cell lines were grown in Dulbecco's MEM containing 4.5 g of glucose, 50 ml each of fetal and newborn bovine sera (Hyclone, Logan, UT), 10 units of penicillin, and 10 μg of streptomycin (Sigma, St. Louis, MO) per liter. Ascitic fluids were produced in pristane-primed mice by intraperitoneal injection of 5×10^5 hybridoma cells in PBS. The mice were pristane primed by intraperitoneal injection with 0.5 ml of 2,6,10,14-tetramethylpentadecane (Sigma, St. Louis, MO) at least 2 wk before injection of the hybridoma cells. A Hyclone mouse monoclonal subisotyping ELISA kit (Hyclone, Logan, UT) was used to isotype the mAb.

Polyclonal Antibody Production

The immunization protocol was described in detail by Kenison et al. (1990). Briefly, a New Zealand white female rabbit was injected intradermally with 15 μg of r-boTNF-α in Freund's complete adjuvant diluted 1:1 with sterile saline. Two weeks later the immunization was repeated. Because the anti-TNF-α antibody titer was low, the rabbit was boosted twice with 1 ml of

Freund's complete adjuvant containing 62.5 and 15 μg, respectively, r-boTNF-α conjugated to itself with gluteraldehyde.

Screening for Production of Anti-TNF Antibodies

Immulon 2 flat-bottom, 96-well plates (Dynatech Laboratories, Chantilly, VA) were coated with 100 μl of r-boTNF-α, 0.2 μg/ml in 0.1 M carbonate-bicarbonate buffer (pH 9.6), and incubated at 4°C for 16 h. After washing the plates three times with PBS containing 0.01% Tween 20, mouse sera (1:10, 1:100, 1:1000 in PBS) or cell culture supernatants (undiluted) were added in 100-μl aliquots, and incubated at ambient temperatures for 2 h. After washing as described, 100 μl of goat anti-mouse IgM, IgG, and IgA conjugated to horseradish peroxidase (Kirkegaard and Perry, Gaithersburg, MD) 1:2000 dilution in PBS with 0.01% Tween 20 were added. The plates were incubated for 2 h at ambient temperature. After washing the plates three times with PBS containing 0.01% Tween 20, horseradish peroxidase activity was detected with a BioRad ABTS substrate kit 2,2'-azino-di[3-ethyl-benzthiazoline-6-sulfonic acid] (Hercules, CA), following the manufacturer's instructions. Absorbance readings were taken using dual filters at 405 and 620 ηm using a Titertek Multiskan (Flow Labs, McClean, VA) plate reader.

Development of an ELISA to Detect TNF-α

The mAb ascites (2C4 and 22D3) and rabbit polyclonal serum were titrated to determine the optimum antibody concentrations by ELISA with r-boTNF-α (400 pg/100 μl) used as the antigen. This concentration was chosen because it was a medium value of the potential serum TNF-α concentrations. The optimum dilution of ascites containing anti-r-boTNF-α antibodies was 1:1000. The optimum dilution of rabbit polyclonal serum was 1:5000. Absorbance values were higher when the ascites induced by cell line 2C4 was used, compared with the 22D3-induced ascites. Therefore, for the ELISA to measure milk whey and blood serum TNF-α concentrations, the ascites containing the antibody secreted by cell line 2C4 was used.

For the ELISA, Immulon 2 flat-bottom 96-well plates (Dynatech) were coated with 100 μl of ascites, containing anti-r-boTNF-α antibodies, diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6), and incubated at 4°C for 16 h. Ascitic fluid produced in mice injected with cell line 2C4 was used. The plates were washed three times with PBS containing 0.01% Tween 20, and 100 μl of 1% BSA in PBS were added to each well. The plates were incubated for 1 h at 37°C in a humidified

chamber. After washing the plates as before, undiluted and diluted (1:5 or 1:20) skimmed milk samples (100 μl) were loaded into each well, and incubated for 2 h at 37°C. Log dilutions of r-boTNF-α (400 to 0.04 ηg per 100 μl in PBS) were included on each plate to standardize absorbance readings, and to determine the detection limits of the assay. After incubation with skimmed milk or r-boTNF-α, the plates were washed 3 times. Rabbit anti-r-boTNF-α polyclonal serum was diluted in PBS containing 1% BSA and 100 μl was added to each well. The plates were incubated for 2 h at 37°C in a humidified chamber. After washing the plates, 100 μl of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) was added to each well. The conjugated antibody was diluted in PBS containing 0.01% Tween 20 to the manufacturer's recommended concentration (1:3000). The plates were incubated for 2 h at 37°C in a humidified chamber, and then washed as before. Horseradish peroxidase activity was detected with a BioRad ABTS substrate kit (2,2'-azino-di-(3-ethylbenzthiazoline-sulfonate, Hercules, CA), following the manufacturer's instructions. Substrate color developed at ambient temperature for 15 min. Absorbance was measured at 410 ηm using a BIO-TEK Instrument (Winooske, VT) EL312 microplate reader. Wells that received PBS instead of mAb served as blanks. The concentrations of TNF-α were calculated by referring to the standard curve.

Cows Used in *Escherichia coli* Lipopolysaccharide Studies

The secretion of TNF in response to intramammary injections of 0.1 and 0.5 mg *Escherichia coli* LPS (0128:B12, Difco Laboratories, Detroit, MI) and to repeated injections of 0.1 mg of LPS was determined. Eight clinically normal Holstein cows in various stages of lactation ($\bar{x}$ = 106 d, range 10 to 180 d) were selected. Cows were free of IMI with foremilk SCC of less than 100,000/ml. Aseptically collected foremilk samples were collected for diagnostic bacteriology (Harmon et al., 1990) and milk SCC (Miller et al., 1986) weekly for 3 consecutive weeks before the start of the experiment and at weekly intervals throughout the study.

Endotoxin Injections

A suspension of either 0.1 or 0.5 mg/ml of LPS in 0.85% saline solution was sterile-filtered (Costar 500-ml bottle top 0.2-μm filter). Right rear mammary quarters of eight cows were injected with 0.1 or 0.5 mg of the LPS preparation suspended in 10 ml of 0.85% saline solution after the morning milking. Four cows were injected with 0.1 mg of LPS and four cows with 0.5 mg

of LPS. Noninjected contralateral quarters served as controls. Foremilk from LPS injected and control quarters, and blood from the jugular vein (via indwelling cannula inserted 24 h before injection) were collected 1.5 and 0.5 h before, at the time of injection, and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h postinjection.

One month later four cows from the previous study were used to study effects of repeated injections of 0.1 mg of LPS. Cows were clinically normal, and milk SCC for all quarters was less than 100,000/ml. All left rear quarters were injected with 0.1 mg of LPS. At 24 h, two cows were again injected in the left rear quarters, and two cows were injected in different right front quarters with the same dose of LPS. The left front quarters served as noninjected controls. Foremilk from control and LPS-injected quarters and blood from the jugular vein were collected 1.5 h before, at the time of injection, and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h, after first and second injections. For all of the experiments, rectal temperatures were obtained at the time of sampling.

Sample Preparation

A portion of the milk samples was used to determine total SCC using an automated cell counter (Fossomatic model 215, Foss Food Technology, Hilleroød, Denmark). Another portion was centrifuged (6500 $\times$ g for 30 min at 4°C) to remove the cream and cell pellet. The sample was recentrifuged, and the residual cream was removed by aspiration. The skimmed secretion was decanted carefully to avoid disturbing the sediment. Samples were centrifuged (46,000 $\times$ g for 30 min at 4°C) to remove casein. The clear supernatant was stored at –20°C until assayed within 1 wk by an ELISA for TNF-α.

At the time of blood collection, one sample was collected in a Vacutainer tube containing EDTA (Becton Dickinson, NJ) as the anticoagulant. Total leukocyte counts were enumerated using an electronic cell counter (Coulter Electronics Inc., Hialeah, FL). Another blood sample was collected in a Vacutainer tube containing no anticoagulant (Becton Dickinson, NJ). After allowing the blood to clot at room temperature, the tubes were refrigerated at 4°C, and the serum was collected over the next 6 h. Serum was centrifuged (6500 $\times g$ for 30 min at 4°C) to remove red blood cells. Serum was stored at –20°C until assayed by an ELISA for TNF-α within 1 wk.

Statistical Analysis

All assays were performed in duplicate, and group means were calculated. Nonlinear and linear regression analyses of the ELISA data were performed with SAS for the PC version 6.03 (SAS Institute Inc., 1987). Milk SCC were converted to natural logarithm scale for statistical analysis and are presented as geometric means in the text (also in Figures 2, 7, and 8 below).

RESULTS

Enzyme-Linked Immunosorbant Assay for TNF-α

Cell lines 2C4 and 22D3 secreted IgG$_1$ and IgG$_{2a}$ antibodies, respectively. The absorbance readings obtained with the 1:1000 dilution of ascites (absorbance = 0.838 for 2C4 or 0.733 for 22D3) were almost identical to those observed using the ascites at a 1:500 dilution (absorbance = 0.873 for 2C4 or 0.733 for 22D3), but the readings were higher ($P < 0.05$) than those obtained with the 1:5000 dilution of ascites (absorbance = 0.434 for 2C4 or 0.379 for 22D3). Therefore, the optimum dilution of ascites containing anti-r-boTNF-α antibodies was 1:1000. Absorbance increased approximately 0.1 when the rabbit polyclonal serum concentration was increased to 1:5000 from 1:10,000. Consequentially, the optimum dilution of rabbit polyclonal serum was 1:5000.

To insure the chosen ascites and serum concentrations were optimal for the entire range of potential TNF-α concentrations, log dilutions of r-boTNF-α (40 to 0.004 ηg) were analyzed using dilutions of each ascites (1:500 and 1:1000) and the rabbit serum (1:5000, 1:10,000, 1:20,000, and 1:40,000). Absorbance values for each r-boTNF-α concentration detected with either dilution of ascites were not different ($P > 0.05$). Therefore, the higher dilution (1:1000) was chosen. The highest absorbance values were obtained using the rabbit serum at 1:5000. Absorbance values were higher when the ascites induced by cell line 2C4 was used, compared with the 22D3-induced ascites. Therefore, for the ELISA to measure whey and plasma TNF-α concentration, the ascites containing the antibody secreted by cell line 2C4 was used.

Using the optimal dilutions of the reagents, the ELISA detected 40 pg of r-boTNF-α in 100 μl; however, 4 pg/100 μl was not detected. Therefore the sensitivity of the assay is at least 40 pg/100 μl. Absorbance measured at 410 ηm was directly related to the concentration of r-boTNF-α. As the r-boTNF-α concentration increased, the absorbance increased.

In Vivo Response to 0.5 and 0.1 mg of LPS

There was no difference ($P > 0.05$) in the TNF-α response in whey after IMI of either 0.1 or 0.5 mg LPS (Figure 1). The TNF-α response started to increase ($P < 0.05$) around 1 h after LPS injection and reached highest values between 2 and 8 h after injection. During
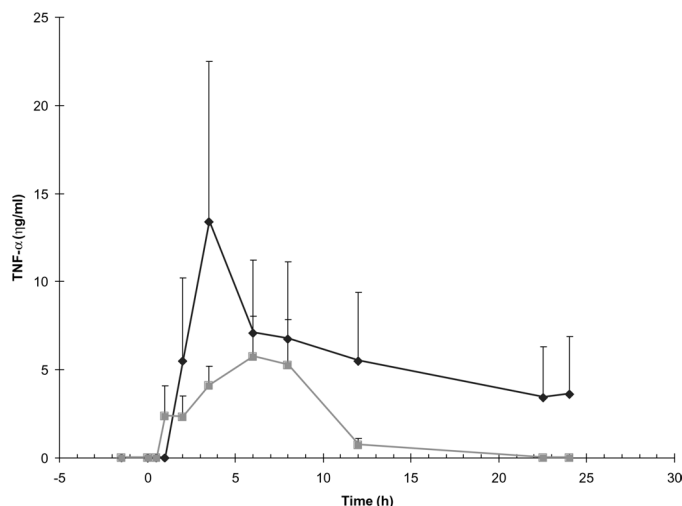
Figure 1. Mean concentrations of TNF-α in whey prepared from quarters injected at 0 h with either 0.1 ($\square$) or 0.5 ($\blacklozenge$) mg of endotoxin. Values represent mean ± SEM of four cows.

Figure 3. Circulating leukocyte counts for cows following intramammary injection at 0 h with either 0.1 ($\square$) or 0.5 ($\blacklozenge$) mg of endotoxin. Each value represents mean ± SEM of four cows.

these times, TNF-α concentrations averaged 4.1 and 7.6 ηg/ml of whey for cows injected with 0.1 and 0.5 mg of LPS, respectively. There were no changes ($P > 0.05$) in TNF-α concentrations in whey from control quarters or in blood serum compared with preinjection concentrations of 0.1 ηg/ml (data not shown).

Milk SCC response to injections of either 0.1 or 0.5 mg LPS were similar ($P > 0.05$, Figure 2). Milk SCC started to increase ($P < 0.05$) at 6 h after injection and reached highest concentrations between 8 and 24 h. During these times, SCC for quarters injected with 0.1 and 0.5 mg of LPS averaged 4.8 and 6.5×10^6 cells/ml of milk, respectively. Milk SCC for control quarters
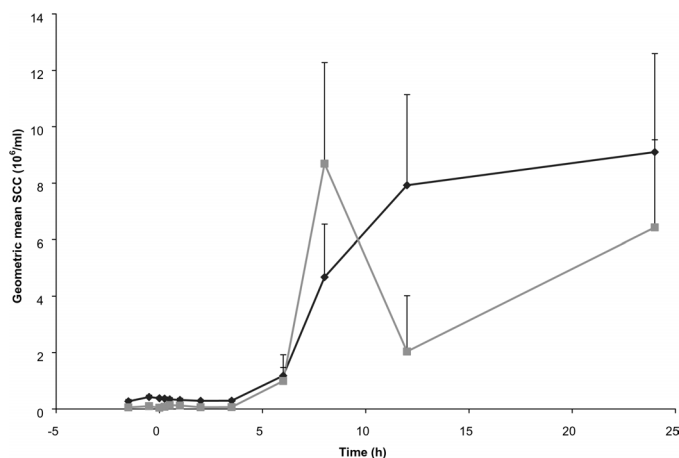
averaged 30×10^3/ml at the time of injection and increased ($P < 0.05$) to 300×10^3/ml at 12 h and remained elevated for the remainder of the sampling times (data not shown).

Circulating leukocytes started to decrease ($P < 0.05$) 1 h after injection of LPS (Figure 3). Lowest concentrations were observed between 6 h after injection of 0.1 and 0.5 mg of LPS averaging 5200 and 5800/mm^3 of blood, respectively. Differences ($P < 0.05$) existed among cows in circulating leukocytes before and after injection of LPS. Some of the difference between the two groups in response to LPS may be attributed to differences ($P < 0.05$) in circulating leukocyte concentrations between the two groups that existed before injection of LPS.

Rectal temperatures increased ($P < 0.05$) after injection of LPS with peak responses occurred between 2 and 6 h after injection (Figure 4). Body temperatures returned to preinjection values by 24 h.

In Vivo Response to Repeated Injections of 0.1 mg of LPS

Concentrations of TNF-α in whey were maximal ($\bar{x}$ = 8.6 ηg/ml) 2 h after the first injection of 0.1 mg of LPS and differed ($P < 0.05$) from preinjection baseline values (Figure 5). Concentrations of TNF-α returned to baseline values by 24 h. A second injection into the same quarters produced a smaller ($P < 0.05$) peak response ($\bar{x}$ = 2.8 ηg/ml) 2 h after injection (26 h after the first injection) compared with the response obtained after the first injection. The second response differed ($P < 0.05$) from baseline concentrations (Figure 5). When LPS was injected twice into different mammary quar-
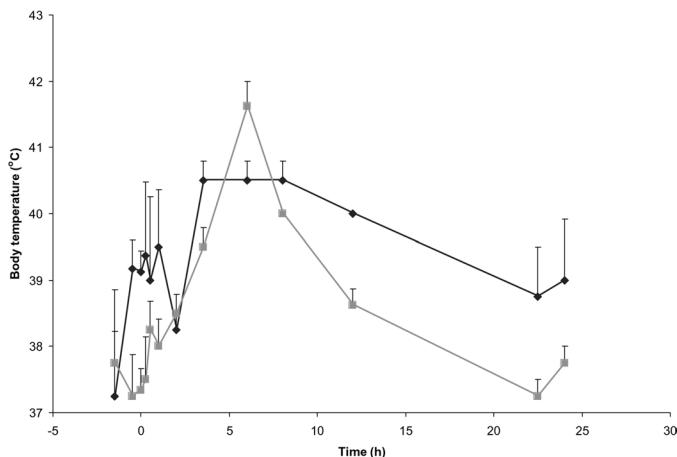

Figure 2. Milk SCC for mammary quarters injected at 0 h with either 0.1 ($\square$) or 0.5 ($\blacklozenge$) mg of endotoxin. Values represent mean ± SEM of four cows.

Figure 4. Rectal temperatures of cows relative to intramammary injection at 0 h with either 0.1 (□) or 0.5 (◆) mg of endotoxin. Each value represents mean ± SEM of four cows.

Figure 6. Mean concentrations of TNF-α in whey prepared following injection of 0.1 mg of endotoxin into left rear mammary quarters at 0 h (◆) and whey from the right front quarters injected with 0.1 mg of endotoxin at 24 h (□). Each value represents mean ± SEM of two cows.

ters TNF-α response did not diminish after the second injection (Figure 6). After the first and second injections, peak responses occurred between 4 to 6 h and averaged 11.0 and 9.1 ηg/ml whey and did not differ from each other ($P > 0.05$). There were no detectable changes ($P > 0.05$) in TNF-α concentrations in whey from control quarters or in blood serum after any of the LPS injections (data not shown).

Milk SCC increased ($P < 0.05$) 6 h after the first injection of LPS and reached maximal concentrations ($\bar{x} = 22,000 \times 10^3$/ml) between 8 and 24 h after injection (Figure 7). In response to the second injection of LPS, SCC initially decreased then increased to concentrations similar ($P > 0.05$) to those observed after the first injection. Part of the decrease in SCC may be attributed
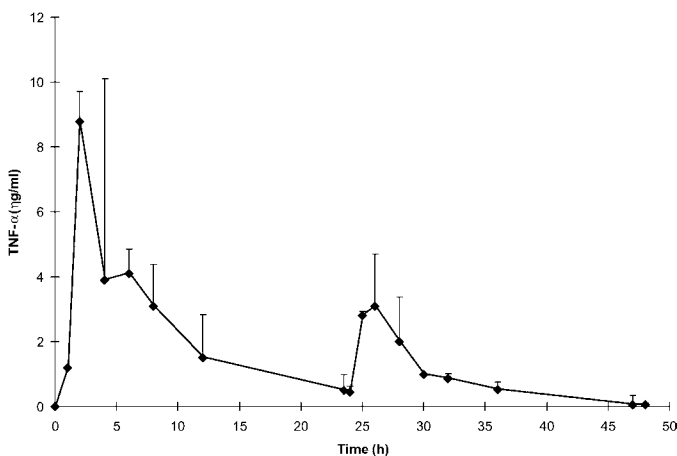
to milking that occurred just before the second injection of LPS. Milk SCC for control quarters averaged 30 × 10^3/ml before injection, increased ($P < 0.05$) to 220 × 10^3 by 24 h, and remained elevated for the duration of the study (data not shown). Injections of LPS into different mammary quarters are shown (Figure 8). After the first injection, a peak SCC response ($\bar{x} = 13,300 \times 10^3$/mL) occurred between 12 to 24 h. After milking at 24 h, SCC decreased and then increased to the before milking SCC by 48 h. After injection into different mammary quarters, peak responses occurred ($\bar{x} = 22,000 \times 10^3$) at 48 h after injection. Control quarters averaged 50×10^3/ml before injection, increased ($P < 0.05$) to 245

Figure 5. Mean concentrations of TNF-α in whey prepared from left rear mammary quarters injected twice with 0.1 mg of endotoxin at 0 and 24 h. Each value represents mean ± SEM of two cows.
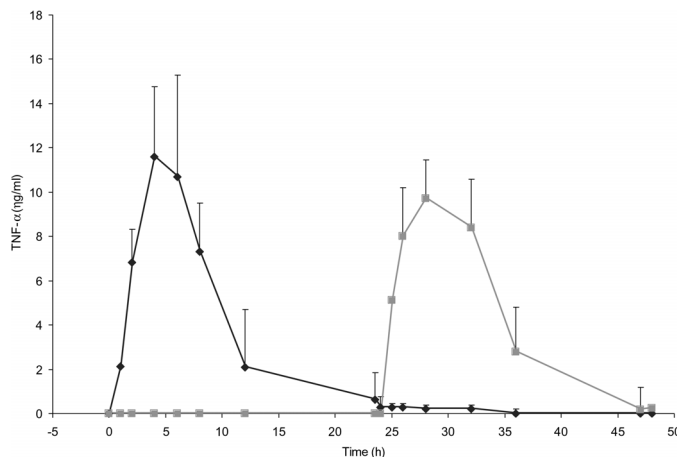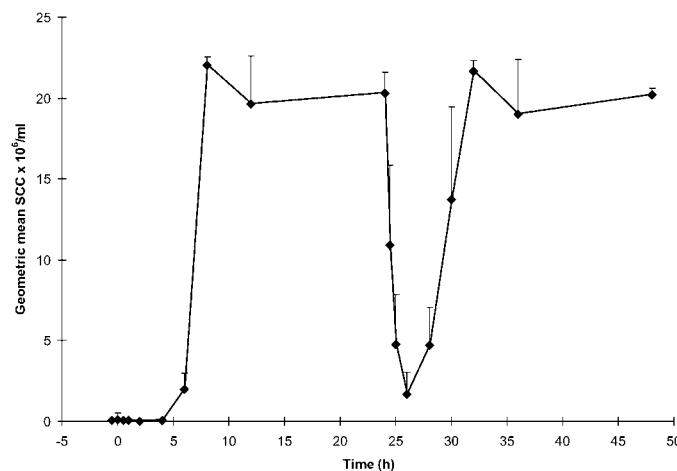

Figure 7. Milk SCC following two injections of 0.1 mg of endotoxin into left rear mammary quarters at 0 h and 24 h. Each value represents mean ± SEM of two cows.
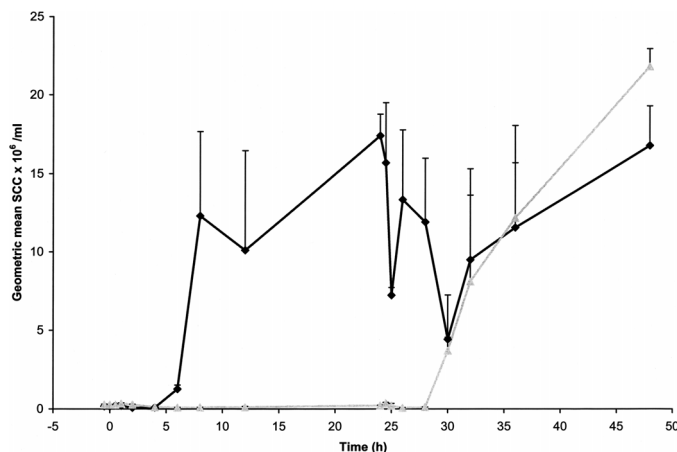
Figure 8. Milk SCC following injection of 0.1 mg of endotoxin into left rear mammary quarters at 0 h ($\blacklozenge$) and in milk from right front mammary quarters injected with 0.1 mg of endotoxin at 24 h ($\triangle$). Each value represents mean ± SEM of two cows.

$\times 10^3$/ml 24 h after the first injection (time of the second injection into) and 469×10^3 24 h after the second injection. Milk SCC for control quarters within the udder of quarters that received the second injection of LPS averaged 80×10^3/ml at the time of the first injection and increased ($P < 0.05$) to 222×10^3/ml by the time of the second injection (data not shown).

Circulating leukocyte counts after the first and second injections of LPS for all cows started to decrease ($P < 0.05$) 2 h after both injections ($P < 0.05$) and reached lowest concentrations between 4 to 6 h (Figure 9). A greater decrease was observed for cows that received
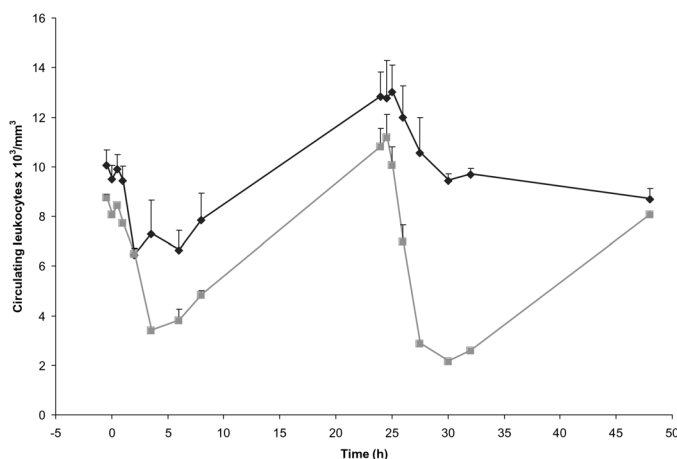

Figure 9. Circulating leukocyte counts for cows following intramammary injections of 0.1 mg of endotoxin into the same mammary quarter at times 0 and 24 h ($\blacklozenge$) and with 0.1 mg of endotoxin into different mammary quarters at times 0 and 24 h ($\square$). Each value represents mean ± SEM of two cows.

the second injection of LPS into different mammary quarters than for cows that received the second injection into the same mammary quarters. The greater decrease may be due to additional chemotactic factors generated in those quarters that did not receive a previous injection of LPS.

Body temperatures for all cows averaged 38°C before injection, started to increase ($P < 0.05$) by 1 h after injection, peaked at 39.7°C by 6 h, and returned to baseline by 12 h (data not shown).

DISCUSSION

TNF-α concentration has been determined with various cytotoxicity assays using L929 or WEHI-164 mouse fibrosarcoma cell lines (Espevik and Nissen-Meyer, 1986). However, the sensitivity of the cell lines to TNF-α lysis can change with in vitro cell passage (Jeffes et al., 1991). The assays are also sensitive to a synergistic effect between LPS and TNF-α (Pfister et al., 1992); consequently, the concentration of TNF-α in samples containing LPS can be overestimated. Commercially available ELISA kits detect human and murine TNF-α; however, the kits do not react well with bovine TNF-α. A radioimmunoassay for bovine TNF-α has been described (Kenison et al., 1990). However, the assay has inherent problems from using radioisotopes including: licensing, storage, disposal, and health hazards. Ellis et al. (1993) described a capture ELISA for ruminant TNF-α, and showed that it correlated well to cytotoxic assays. The ELISA developed in this study correlated well with a radioimmunoassay for bovine TNF-α (Malstrom et al., 1994) and can be an alternative choice to the above assays for measuring bovine TNF-α.

The observation that there were no differences in the TNF-α and inflammatory responses after injections of either 0.1 or 0.5 mg of LPS suggests that the 0.1-mg dose provided the maximum responses. In previous studies, intramammary injection of 0.010 and 0.033 mg induced a local inflammation without detectable increases in milk TNF-α (Rainard and Paape, 1997; Shuster et al., 1993). This suggests that a strong inflammatory reaction is necessary to attain detectable amounts of TNF-α in milk.

A second injection of 0.1 mg of LPS, when the initial local inflammation had not yet abated, maintained the local manifestations of inflammation, and evoked a lesser TNF-α response in milk than that observed after the first injection of LPS. A possible explanation could be the presence of large quantities of soluble CD14 (sCD14) in milk that were shed from the recruited neutrophils. Bovine neutrophils contain a large intracellular pool of CD14 that is translocated to the surface when neutrophils migrate into milk (Paape et al., 1996). It is

recognized that two forms of CD14 exist, a membrane and soluble form (Maliszewski, 1990). The soluble form results from the shedding of membrane CD14 (mCD14). The mCD14 receptor is a 53-kDa phosphoinositol-linked protein, whereas the sCD14 is a 43-kDa protein. The mCD14 pathway involves LPS binding protein (**LBP**), a 60-kDa glycoprotein synthesized in hepatocytes during the APR in the cow (Khemalni et al., 1994). Release of TNF-α is attributable to binding of the LPS-LBP complex to mCD14. The LBP is not required for the binding of LPS to sCD14. Thus, sCD14 can bind LPS directly and prevent it from binding to and activating mCD14. We have reported that sCD14 is present in bovine milk (Wang et al., 1997). It is conceivable that the massive somatic cell response, of which 98% can be assumed to be neutrophils (Paape et al., 1996), resulted in an abundance of sCD14 in milk, which competed with LBP for LPS and partially prevented LPS-LBP from interacting with mCD14. Despite the attenuated TNF-α response, increases in SCC and body temperature were similar to that observed after the first LPS injection, where a greatly elevated TNF-α response was observed. This indicates that migration of neutrophils from blood to milk and body temperature were not regulated by TNF-α. Release of other cytokines by mammary cells after exposure to LPS probably contributed to the inflammatory response.

Mammary quarters that were not exposed to a previous injection of LPS but were adjacent to a quarter that received an injection of LPS 24 h earlier, produced a normal TNF-α response when injected with LPS. This indicated that the attenuation of the TNF-α response in quarters to a second injection of LPS, was a local and not a systemic response. A possible explanation for the responsiveness of adjacent quarters to LPS could be due to the low concentration of sCD14 in milk, and a greater macrophage to neutrophil ratio in adjacent quarters before the initial injection.

In contrast to the rapid TNF-α response to 0.1 mg of LPS, we have shown that a low dose of LPS (0.033 mg) will not produce a TNF-α response or increase in body temperature when injected into a mammary quarter (Rainard and Paape, 1997). However, a second injection into the same quarter or into adjacent quarters produced a TNF-α response in all injected quarters and an increase in body temperature. This indicates that after an initial contact with a low dose of LPS, the whole mammary gland became more responsive to a second stimulation. Taken together with the observations from the present study, this suggests that the regulation of TNF-α at the level of the mammary gland may play an important role in the regulation of the inflammatory response to LPS. An excess of TNF-α production could result in an exaggerated inflammatory response lead-

ing to excessive tissue damage or even death of the animal (Sordillo et al., 1991). Too low a level of LPS, while resulting in leukocytic infiltration, will not cause TNF-α release or a fibril response, thus minimizing possible tissue damage. The resulting low-level mobilization of leukocytes may be sufficient in clearing the invading coliform organisms. If not, growth of the organisms would produce additional LPS, which would then exceed sCD14 levels in the milk, resulting in release of TNF-α and augmentation of leukocytic infiltration and initiation of the fibril response. How an LPS challenge in one mammary quarter sensitizes other mammary quarters to a subsequent LPS could be explained by the presence of LBP as a result of the modest inflammatory response in those quarters.

CONCLUSIONS

Secretion of TNF-α is down-regulated in response to repeated intramammary injections of endotoxin into a mammary quarter. This event may be important in controlling the extent of the inflammatory response and subsequent mammary tissue damage.

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